09/733,485

PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 15/01, C07K 14/47, A61K 38/17 — A2

(11) International Publication Number: **WO 97/30150**

(43) International Publication Date: 21 August 1997 (21.08.97)

(21) International Application Number: PCT/EP97/00777

(22) International Filing Date: 17 February 1997 (17.02.97)

(30) Priority Data:
96200383.6 15 February 1996 (15.02.96) EP
(34) Countries for which the regional or international application was filed: AT et al.

(71) Applicant *(for all designated States except US)*: PANGENETICS B.V. [NL/NL]; Paasheuvelweg 15, NL-1105 BE Amsterdam (NL).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: BALINT, Robert, F. [US/US]; 4003 Scripps Avenue, Palo Alto, CA 94306 (US). WAUBEN, Marca, Henriëtte, Michaela [NL/NL]; Hollywoodlaan 101, NL-1325 HP Almere (NL).

(74) Agent: VAN SOMEREN, Petronella, Francisca, Hendrika, Maria; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
Without international search report and to be republished upon receipt of that report.

(54) Title: MOLECULES FOR THE INDUCTION OF IMMUNOLOGICAL TOLERANCE

(57) Abstract

The invention relates to a population of protein molecules having a distribution of specific mutations in the amino acid sequence as compared to a parent protein, which population of protein molecules is obtainable by establishing the parental amino acid sequence of the protein to be mutated and deriving therefrom a parental nucleotide sequence encoding the amino acid sequence, optionally selecting sites within this parental amino acid sequence, mutation of which is more desirable or less desirable, designing overlapping oligonucleotides encoding parts of the protein and harbouring one or more codon changes as compared to the corresponding parental nucleotide sequence, composing of the overlapping oligonucleotides a population of mutated nucleotide sequences encoding mutated versions of the parent protein thus establishing a library, and expressing the mutated versions of the mutated nucleotide sequences to obtain a population of mutated proteins.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AM	Armenia	**GB**	United Kingdom	**MW**	Malawi
AT	Austria	**GE**	Georgia	**MX**	Mexico
AU	Australia	**GN**	Guinea	**NE**	Niger
BB	Barbados	**GR**	Greece	**NL**	Netherlands
BE	Belgium	**HU**	Hungary	**NO**	Norway
BF	Burkina Faso	**IE**	Ireland	**NZ**	New Zealand
BG	Bulgaria	**IT**	Italy	**PL**	Poland
BJ	Benin	**JP**	Japan	**PT**	Portugal
BR	Brazil	**KE**	Kenya	**RO**	Romania
BY	Belarus	**KG**	Kyrgystan	**RU**	Russian Federation
CA	Canada	**KP**	Democratic People's Republic of Korea	**SD**	Sudan
CF	Central African Republic			**SE**	Sweden
CG	Congo	**KR**	Republic of Korea	**SG**	Singapore
CH	Switzerland	**KZ**	Kazakhstan	**SI**	Slovenia
CI	Côte d'Ivoire	**LI**	Liechtenstein	**SK**	Slovakia
CM	Cameroon	**LK**	Sri Lanka	**SN**	Senegal
CN	China	**LR**	Liberia	**SZ**	Swaziland
CS	Czechoslovakia	**LT**	Lithuania	**TD**	Chad
CZ	Czech Republic	**LU**	Luxembourg	**TG**	Togo
DE	Germany	**LV**	Latvia	**TJ**	Tajikistan
DK	Denmark	**MC**	Monaco	**TT**	Trinidad and Tobago
EE	Estonia	**MD**	Republic of Moldova	**UA**	Ukraine
ES	Spain	**MG**	Madagascar	**UG**	Uganda
FI	Finland	**ML**	Mali	**US**	United States of America
FR	France	**MN**	Mongolia	**UZ**	Uzbekistan
GA	Gabon	**MR**	Mauritania	**VN**	Viet Nam

MOLECULES FOR THE INDUCTION OF IMMUNOLOGICAL TOLERANCE

The present invention relates to a method for producing and selecting one or more new protein molecules for use in the treatment of autoimmune diseases. The invention further relates to the protein molecules thus produced and/or selected and their use in therapy and/or prophylaxis.

Most diseases are associated with tissue or cell damage. In many cases of such damage, the liberation of (intracellular) antigens will lead to activation of the immune system. This situation is potentially harmful for our body, since it may lead to an inflammatory response. In the case of infectious diseases such inflammatory responses are needed in order to create an environment which is hostile to the invading microorganism. In most infectious diseases the inflammatory process is leading to accompanying immunopathology, which is normally kept within bounds by the self-regulating capacity of the immune response. The mechanisms operative in this self-regulation are far from being understood at present. It is generally accepted, however, that the self-regulatory mechanisms as operative in peripheral tolerance (see under) can become compromised, which then may result in the development of a so-called autoimmune disease.

The immunopathology seen in autoimmune diseases, diseases in which the immune system primarily attacks "self-tissues", resembles the immunopathology seen in inflammation resulting from infection. Autoimmune diseases are usually chronic and cause major socio-economic losses. Examples of autoimmune diseases are rheumatoid arthritis, multiple sclerosis, type I diabetes, lupus, thyroiditis and myasthenia gravis. Since in these diseases "self-proteins" are being recognised by the immune system, the origin of autoimmune diseases is being thought to reside in a defective self-regulation, or incomplete tolerance to such

self-proteins. These self-proteins are therefore called autoantigens. It is possible that the break of tolerance to self-proteins is elicited by contact (possibly infection) with certain microorganisms. The critical question is how in the case of autoimmune diseases the self-regulatory mechanisms of the immune response fail.

Multiple sclerosis is a severely disabling progressive neurological disease involving autoimmune attack against myelin in the central nervous system. MS affects 1 in 1000 persons in the USA and Europe. Due to improved diagnosis that number is currently increasing. Onset of disease is usually around 30 years of age and, on average, patients are in need of treatment for another 28 years. Costs of MS treatment and the long term hospitalization of these patients are enormous and place serious constraints on public health budgets. MS is among the most expensive chronic diseases of western society based on duration and intensity of care. The etiology of the disease is still essentially unknown hampering development of preventive strategies. On the other hand, diagnosis of exacerbations and early identification of onset of exacerbations has improved greatly, allowing design of novel treatment strategies.

Optimal therapeutic strategies for the treatment of autoimmune diseases, such as those mentioned above, should be based on understanding the underlaying cellular and molecular mechanisms leading to the disease. Until recent years is was generally thought that the immune system was directed to discrimination between self and nonself. Selection of an immunological repertoire of T-cells was found to occur in the thymus, in such a way that cells capable of responding to self-proteins were deleted from the system. The remaining repertoire of cells were those lymphocytes having receptors for recognising non-self antigens.

However, now it has become clear that this process of selection is incomplete and that in fact the immune repertoire is containing a great number of cells

which have the intrinsic capacity of responding to self-proteins or autoantigens. This perception has led some investigators to suppose that self-reactivity is one of the major elements of the normal functioning immune system.

As an inescapable consequence of this new perception, the focus of research has shifted from self-nonself discriminatory processes into mechanisms of internal regulation for maintaining peripheral (in contrast to "central" as imposed by thymic selection) tolerance. Such mechanisms of peripheral tolerance include anergy induction at the level of individual "anti-self" T-cells, regulatory (inhibitory) T-cell-T-cell interactions and production of inhibitory cytokines by specialised T-cell subsets leading to (by-stander) suppression of inflammatory cells.

It is now well established that T-cell responses may vary almost continuously from full activation to anergy or even cell death, and also with respect to cytokine profile, depending on the strength and/or quality of the TcR/peptide-MHC interaction (Evavold et al., 1993; Sette et al., 1994; Paul and Seder, 1994; Janeway, 1995; Chien and Davis, 1995).

Originally, affinity was believed to be the sole criterion for antigen-specific T-cell activation. It is now clear, however, that other factors, such as antigen density and ligand-induced conformational effects, are equally important as determinants of the T-cell response (Janeway, 1995). Thus, analogs of peptides that normally fully activate a given T-cell could antagonize, anergize, or even kill the same T-cell, or alter its cytokine phenotype. As a result, there is currently considerable interest in the potential of antagonistic and/or tolerogenic peptide analogs for the treatment of autoimmune and allergic diseases.

T-cell signaling through the TcR is a complex process involving both serial and parallel sets of events, initiated by ligation of TcR with specific

peptide/MHC complexes on the antigen presenting cell (APC) to generate the T-cell/APC "synapse" (Paul and Seder, 1994). Aggregation of multiple TcR-peptide/MHC complexes at the T-cell/APC interface then leads to or coincides with cooperative activation of at least two autonomous modules, CD3$\gamma\delta\epsilon$ and the CD3γ chains. These events are followed by up-regulation of costimulatory surface molecules, endocytosis of the TcR, and the full cascade of T-cell effector functions, including proliferation and cytokine production. The CD3γ chain may be phosphorylated at up to six sites in response to TcR ligation, and subtleties in the latter can allosterically affect the specific pattern of phosphorylation, which in turn can determine specific allosteric effects on downstream effectors. Thus, T-cell responses are exquisitely sensitive both quantitatively and qualitatively to subtle alterations, both quantitative and qualitative, in the TcR-peptide-MHC interaction.

Antigen analogs have been used successfully to dissect out component parts of the T-cell activation process (e.g., Evavold and Allen, 1991). Most of the analogs studied differed from the antigen conservatively at one or two positions, and bound to MHC with comparable affinities. Most of the analogs were partial agonists in that they activated some but not all T-cell effector functions. Furthermore, like antagonists, most partial agonists had a net inhibitory effect on T-cell activation, and could therefore be considered a subset of the former.

TcR antagonism by antigen analogs was first reported by De Magistris et al. (1992)., who showed that single-site analogs could inhibit antigen-specific activation of T-cells under conditions in which direct competition for MHC was ruled out by pre-loading APCs with antigen. The requirement for direct TcR contact by the analogs was clearly indicated by the idiotype-specificity of the effect. Since they could find no evidence of anergy induction or other negative signaling,

De Magistris et al. speculated that the inhibition might have resulted from direct competition between antigen/MHC and analog/MHC complexes for limiting TcR.

Other reports have also ruled out competition for MHC (e.g., Ostrov et al., 1993), but there is as yet no direct evidence for or against competition for TcR. Janeway (1995) has pointed out that, in vivo, ligation of only a tiny fraction of the ~50,000 TcR molecules on a T-cell is typically required for signaling, and it does not seem likely that an APC would ever naturally present enough of any single peptide antigen or analog to effectively compete for that many TcR. Thus, most antigen analogs do not appear to antagonize by direct competition for either MHC or TcR.

Recent studies have shown that T-cell activation involves multiple serial TcR ligations by each peptide-MHC complex, and this in turn requires relatively high dissociation or 'off' rates (Valitutti et al., 1995). This suggests that optimum TcR-antigen affinities for T-cell activation must be neither too high nor too low, and that high-affinity analogs might be relatively common and particularly inhibitory. Reports that some natural antigen analogs could antagonize effectively at lower concentrations than antigen, in some cases 10-fold or 100-fold lower (Klenerman et al., 1994; and Bertoletti et al., 1994), suggest that some antagonists may, in fact, have higher TcR affinities than agonists.

Other observations suggest that the correlation between affinity and activation or antagonism may be weak. For example, the ability of anti-TcR antibodies to activate or antagonize T-cells does not correlate with affinity (Yoon et al., 1994). Also, many antigen analogs have differential effects on different T-cell clones from the same polyclonal response with no discernible differences in affinity (Janeway, 1995). Thus, whether a given peptide will antagonize or activate a given T-cell appears to depend on conformational effects which do not necessarily correlate with affinity for the TcR.

The findings described above suggest that antigen-specific antagonism of T-cell activation may be a dominant effect of ligation of the TcR with an MHC-borne antigen analog which fails to induce the necessary conformational changes for full activational signal transduction, but which instead either blocks agonist/TcR signaling by forming non-productive co-aggregates, or transmits incomplete signals which induce anergy or apoptosis (Sette et al., 1994; Janeway, 1995). The former mechanism is reminiscent of trans-dominant mutational effects, in which multisubunit complexes are inactivated by the presence of an inactive mutant subunit, even in the presence of the wildtype allele.

Recent advances in the understanding of T-cell activation are consistent with a trans-dominant mechanism for the inhibition of T-cell activation by non-productive TcR ligation (Sakihama et al., 1995). Cross-linking of TcR-peptide-MHC complexes is essential for T-cell activation. Productive ligation induces conformational shifts in the TcR which lead to the recruitment of the CD4 co-receptor. Upon MHC binding CD4 then undergoes a conformational shift which induces tetramerization, and it is this tetramerization of ligated CD4 which drives cross-linking of the TcR-peptide-MHC complex and consequently T-cell activation. Failure of an antigen analog to induce the necessary conformational changes could inhibit cross-linking, even in the presence of excess wildtype antigen, and thereby prevent activation.

Many dominant antagonists produce no detectable signal transduction effects, implying the involvement of the 'trans-dominant' mechanism. High-affinity analogs may inhibit signaling by inhibiting TcR internalization. But, in heterodimers of TcR-peptide/MHC complexes, they may also trans-dominantly inhibit TcR disengagement from antigen, thereby inhibiting T-cell activation. In other cases, antigen analogs can induce T-cell anergy or even cell death (Sloane-Lancaster et al., 1993), suggesting the involvement of incomplete signaling.

However, cell death may also occur as a result of CD4 or CD8 ligation in the absence of TcR signaling. Ligation of CD4 or CD8 in the absence of TcR ligation has been shown to stimulate expression of both FAS antigen and FAS ligand and to lead to apoptosis (Nagata and Golstein, 1995). Thus, antagonistic ligation of the TcR might also induce apoptosis by this mechanism. In any case, the apparent trans-dominant nature of antigen-specific TcR antagonism has profound implications for the potential efficacy of antigen analogs for the treatment of autoimmune disease.

Antigen-dependent T-cell activation appears to be exquisitely sensitive to the fine structure of the TcR-peptide/MHC complex (Chien and Davis, 1995). Thus, among close antigen analogs the frequency of antagonists may be quite high, and comparable to if not higher than the frequency of agonists. Jameson et al. (1993) have found that more than 40% of 64 close analogs of an ovalbumin peptide were antagonistic for one or more of three specific cytotoxic T-cell clones. Though no single analog antagonized all antigen-specific clones, in a separate study most antagonists were found to be able to antagonize at least half of the antigen-specific T-cell clones (Franco et al., 1994). These results imply that, on average, for any T-cell clone, on the order of 20% of class II presentations of antigen analogs should be antagonistic, and it is likely that a large fraction of the remainder do not bind the TcR. Thus, in such a pool of analogs there are probably at least as many antagonists as agonists. Given the apparent 'trans-dominant' nature of TcR antagonism, it seems reasonable to expect that most APCs presenting a moderate pool of antigen analogs should be capable of antagonizing most of the antigen-specific T-cell repertoire. In the rodent experimental autoimmune encephalomyelitis (EAE) model, Franco et al. (1994) found that a selected pool of antagonists provided potent protection from disease

induced by a single immunodominant peptide, even when co-administered with equimolar antigen.

Accumulating evidence supports an emerging view of peripheral self tolerance as a dynamic balance between partial agonism, i.e. suppression, and full agonism, i.e. inflammatory activation, of anti-self T-cells, in which the former normally dominates (Allen, 1994). A number of observations point to a quantitative relationship between antigen dose and the cytokine profile of responding T-cells. The prevailing view of thymic selection is that T-cell fate is a quantitative function of TcR ligation, that is, it is dependent on the product of TcR-peptide and peptide-MHC affinities, TcR density, and peptide/MHC density on the APC (Allen, 1994; Fairchild and Wraith, 1996). Thus, a high-affinity TcR could be positively selected if the antigen density were low enough (rare or low MHC-affinity), and a low affinity TcR could be negatively selected if the antigen density were either too low or too high. In the periphery, T-cell responses are similarly dependent on quantitative TcR ligation. Thus, whereas weak TcR ligation, as might be produced by self antigens at normal, low concentrations, would have induced positive selection in the thymus, in the periphery it would induce either anergy or differentiation into TH2 cells, which secrete the anti-inflammatory cytokines, IL-4 and IL-10. Above a certain threshold of TcR ligation, determined by the product of affinity and antigen density, the TH1 phenotype would be induced and secretion of IL-2 and IFNγ would promote inflammation. Sub-threshold TcR ligation may favor induction of the TH2 phenotype because the TH2-promoting co-activator B7-2 is constitutive on APCs, whereas the TH1-promoting co-activator B7-1 is not. Above-threshold amounts of TcR ligation may activate the APC, inducing B7-1 expression which in turn induces the Th1 phenotype in the responding T-cell (Kuchroo et al., 1995).

Most autoimmune diseases involve a breakdown of peripheral tolerance in the form of inappropriate TH1

responses to one or more self antigens. This situation can arise in several ways, many of which involve pathological combinations of anti-self TcR affinity and self antigen abundance. As mentioned above, even relatively high anti-self affinities can be positively selected in the thymus on low-abundance or low-MHC-affinity antigens, and low affinities can mediate TH1 induction in the presence of sufficient amounts of antigen. Inflammation, however caused, can result in the accumulation of fully-agonizing, i.e. TH1-inducing, amounts of self antigen. The presence of the well-known stress-induced proteins known as heat shock proteins (hsp) in the thymus makes them likely to be a common template for positive selection, and their abundance at sites of inflammatory stress makes them likely candidates for self antigens which could sustain an inflammatory response (Elias et al., 1995).

Other pathologies such as infection or genetic defects could also induce autoimmunity by promcting the accumulation of fully-agonizing amounts of self antigen or by activating self-cross-reactive T-cells which could acquire a TH1 phenotype in response to normal amounts of self antigen.

Regardless of how autoimmune disease arises, peripheral self tolerance is normally maintained and could presumably be restored, largely by the predominance of regulatory cytokines, primarily IL-4 and IL-10, at sites of T-cell-antigen encounter. Recent evidence suggests an alternative mechanism of peripheral tolerance in which antigen presentation by mature tissue macrophages normally leads to clonal deletion of reacting, i.e. anti-self, T-cells in the absence of inflammation (Munn et al., 1996). In response to e.g. infection, local non-specific inflammatory production of IFNγ may lead to up-regulation of co-activators on APCs and full activation of reacting T-cells, including anti-self T-cells. Therefore, therapeutic strategies which help shift the cytokine balance from inflammatory (e.g.,

IFNγ) to regulatory (e.g., IL-4) have the distinct advantage of mimicking natural immunoregulation. Conservative analogs of inflammatory self antigens promise to do just that by inducing anergy or TH2 differentiation of T-cells which would otherwise mount a pro-inflammatory TH1 response to the cognate self antigens.

In a mouse EAE model (Brocke et al. 1996) conservative analogs of the inducing epitope with reduced affinity for the TcR were found to reverse established autoimmune disease by shifting the cytokine balance from inflammatory (TNF-α) to regulatory (IL-4), suppressing all inflammatory T-cells, even those with specificities unrelated to the tolerogenic analogs. This observation is extremely important because chronic autoimmune disease in humans is characterized by multiple inflammatory epitopes derived from several self proteins, and is highly variable from patient to patient. While a single epitope, different in each case, is probably responsible for initiation of inflammation, with time the inflammatory cytokine milieu promotes the appearance of additional inflammation-promoting anti-self T-cell idiotypes. The latter idiotypes and associated epitopes are largely responsible for maintenance of the chronic inflammatory condition. Though variability is high from patient to patient, it is likely that, for stereotypical autoimmune diseases such as rheumatoid arthritis (RA), insulin-dependent diabetes (IDDM), and multiple sclerosis (MS), there exists a subset of inflammatory epitopes that recurs in most patients. It is also likely that conservative analogs of any of these epitopes could have TH2-promoting, anti-inflammatory effects in these diseases.

On the basis of the observations presented above, it was hypothesized by the present inventors that autoimmune inflammation could be suppressed and peripheral tolerance of the offending self proteins could be induced by parenteral administration of a mixture of

conservative analogs of all epitopes of at least one known inflammatory self protein.

The present invention thus relates to a population of protein molecules having a distribution of specific mutations in the amino acid sequence as compared to a parent protein. Such a population is for example obtainable by establishing the parental amino acid sequence of the protein to be mutated and deriving therefrom a parental nucleotide sequence encoding the amino acid sequence, optionally selecting sites within this parental amino acid sequence, mutation of which is more desirable or less desirable, designing overlapping oligonucleotides encoding parts of the protein and harbouring one or more codon changes as compared to the corresponding parental nucleotide sequence, composing of the overlapping oligonucleotides a population of mutated nucleotide sequences encoding mutated versions of the parent protein thus establishing a library, and expressing the mutated versions of the mutated nucleotide sequences to obtain a population of mutated proteins.

Modifying a complete autoantigen protein instead of all the separate peptides encoding the relevant T-cell epitopes occurring in the autoantigen has a number of specific advantages for therapeutic use in autoimmune diseases. By modifying a complete protein at selected sites, the naturally occurring conformation of the protein remains largely intact and will initially be recognized by the immune system in the same way as the disease causing autoantigen. This will ensure that the same immune cells that are involved in the autoimmune process will see the modified autoantigen. When peptides are used, it is most likely that these will be presented to the immune system in a different way as the original autoantigen, whereby immune cells other than those involved in the autoimmune process will encounter these peptides. An other consequence of this could be that not all immune cells involved in the autoimmune process will be reached by the peptides and thus escape from the

desired therapeutic effect. Using a whole protein makes it possible to incorporate mutations in different T-cell epitopes, thus targeting all the T-cells involved in the autoimmune process with one pharmaceutical composition. Using specific peptides will require the development of more than one peptide that can significantly differ in their pharmacokinetic behavior, solubility and other characteristics important for a pharmaceutical composition. Furthermore, for the development of specific peptides, detailed information on all T-cell epitopes on a given autoantigen is needed before the development of modulatory peptides can be initiated.

The approach described in the present invention allows the initiation of a development program towards the generation of a pharmaceutical composition to treat the autoimmune disease in question in absence of specific knowledge of the offending T-cell epitopes. The present invention thus describes a method for the construction of autoantigen libraries of conservative analogs of the autoantigen, which contain a sufficiently high proportion of antagonists of all possible T-cell epitopes to significantly ameliorate the symptoms associated with the given autoimmune disease. For the construction of these autoantigen analog libraries, a method for oligonucleotide-directed scanning mutagenesis called Parsimonious Mutagenesis (PM) (Balint and Larrick, 1993) was used. This method is known in the art for the optimization of antibody binding affinity and is now used in this invention for the generation of libraries of conservative autoantigen analogs.

Parsimonious Mutagenesis (PM) is a method for the combinatorial optimization of proteins based on the premise that the optimization of a protein for a particular application when only the primary sequence is known can be accomplished by conducting a thorough search of that portion of the sequence space of the protein comprised of variants which contain single-base coding

changes at no more than a few percent of the total positions in the protein.

Natural mutagenesis occurs by the incorporation of errors during DNA of RNA replication. PM works in much the same way, except that the "errors" are incorporated into synthetic oligonucleotides, which are in turn incorporated into protein-coding DNA by the polymerase chain reaction (PCR). In PM, precise control is exercised over which positions are mutagenized, the frequency of mutagenesis at each position, and which nucleotides are used as replacements. This is accomplished during oligonucleotide synthesis by using nucleotide mixtures for each position. Each mixture contains the nucleotide specified by the natural, or parent sequence, "spiked" or "doped" with small, precisely determined, amounts of one or more of the other three nucleotides. When the resulting oligonucleotides are used to synthesize a gene and the gene is expressed in a population of cells, the resulting population of protein molecules has the prescribed frequency of substition at each mutagenized position with a prescribed subset of the other nineteen amino acids.

PM, like natural mutagenesis, produces a binomial distribution of the number of mutations per protein molecule in which molecules with the prescribed frequency comprise the most abundant class. For example, to maximize the frequency of molecules with four amino acid substitutions out of 100 positions mutagenized, the coding sequence would be spiked to produce one base change at each amino acid position (3-base codon) in every 25 molecules. This would produce a binomial distribution in which molecules with exactly four mutations comprise 20% of the library, and molecules with 2-6 mutations comprise 80% of the library. At every amino acid position one of the prescribed subset of substitutes would occur for every 24 occurrences of the parent, or natural, amino acid.

Substitute amino acid sets are specified by using degenerate codons in place of the codons of the parent coding sequence. These degenerate, or "doping" codons specify more than one nucleotide at each position, and this in turn encodes multiple amino acids. For example, the doping codon MRK specifies A or C at the first position, A or G at the second position, and G or T at the third position (IUB code see Table 1) for a total of eight different codons. With lysine as the parent amino acid (AAG), this doping codon would be synthesized to specify three conservative alternates, Arg, Gln, and Asn with equal frequencies. Two additional alternates, His and Ser would be specified at much lower frequencies. Such conservatively mutated sequences allow the library to "probe" the structure of the protein for new properties with minimal disturbance of the properties which are to be retained.

A computer program, called PM-CAD, assists with the design and construction of PM libraries. The program consists of a series of computational spreadsheets and searchable databases, which allow the user to interactively determine the optimum parameters for construction of a library with the highest possible enrichment for desired variants. Based on the parent amino acid sequence, the "most likely" number of amino acid substitutions in the desired variants (usually 5-20% of the total), and the desired sets of alternative amino acids, the program computes the required nucleotide (nt) mixtures for each position to be mutagenized in the oligonucleotides.

The first step in PM library design is to decide on an amino acid substitution frequency, which would allow a thorough search of the protein sequence space without sacrificing essential properties. Next, doping codons are selected for each position on the basis of a user-specified set of alternative amino acids. The program extracts all doping codons which encode the specified set from any of several user-selected

databases, and computes the frequency in the library of each encoded alternate amino acid at the position in question. Once doping codons have been selected for each site, the program computes the required nucleotide mixtures for oligonucleotide synthesis.

Table 1
Nucleic acid codes in doping codons to the IUB standard

Code	Represents
R	G, A
Y	T, C
K	G, T
M	A, C
S	G, C
W	A, T
B	G, T, C
D	G, A, T
H	A, C, T
V	G, C, A
N	A, G, C, T

The population of mutated proteins will inter alia comprise proteins that can influence T-cell activation. This influence can lead to complete abolishment of T-cell activation, to self inactivation of the T-cells or to the induction of suppression factors, like particular cytokines. The induction of suppression factors will ultimately lead to downregulation of the immune response in total. Thus, the influence may also comprise a positive, upregulating or stimulating effect on suppressor T-cells. In this application the terms "antagonise", "antagonist" and "antagonistic" will be used to describe a negative, downregulating or inhibitory influence on T-cell activation either directly or indirectly by stimulating suppressor mechanisms. "Agonise", "agonist" and "agonistic" are used to describe

stimulating effects on T-cell activation by wildtype autoantigen (epitope)s.

The population as such can therefore be used in treatment and prophylaxis. Also the population can be studied further to select antagonistic fractions or sets of antagonistic species therefrom. In a further step, one relevant antagonistic species or even one or more parts of one or more anatagonistic species may be selected.

Selection is performed by studying the ability of the whole population, fractions, sets of species, individual species or parts of species to antagonise the activation of T-cells, either in vitro or in vivo. The T-cells may be monoclonal or polyclonal T-cells and should be specific for the parent protein from which the population of mutated proteins is derived.

In this application the term "population" is intended to encompass the first group of mutated molecules being directly derivable from a complete library. A "fraction" is a subgroup of the population that is selected from the population based on the criterion that it has either of the two types of influences on T-cell activation described above. A "set of species" consists of two or more individual species of the population which have either of the two types of influence described above. An "individual species" is just one species from the population having either of the two types of influence. A "part of a species" is a fragment, domain, subunit and the like of a species of the population having either type of influence on T-cell activation.

In a particular embodiment of the invention a library of conservative analogs is created by PM-scanning of an autoantigenic protein, such as the 21.5 kDa form of myelin basic protein (MBP), the major autoantigen of multiple sclerosis (MS), at a modal frequency of ~1 mutation per 10 aa (MHC class II presents ~10-mers). For any and all 10-mers, fragments with 1 mutation would comprise (10 x 0.1 x 0.99) ≈ 40% of the population and

fragments with 2 mutations would comprise (10!/2!/8! x 0.12 x 0.98) (20% of the population. Thus, ~60% of MHC class II presentations of an autoantigenic peptide will present one or two conservative substitutions in that peptide. If, as discussed above, ~40% of conservative antigen analogs are antagonistic for at least half of the antigen-specific T-cell clones (Jameson et al., 1993; Franco et al., 1994), then, on average, about 20% of MHC class II presentations of conservative analogs of a given epitope should be antagonistic for any T-cell clone specific for that epitope. If at least 60% of a given epitope in a PM library are conservative analogs, then as many as 12% of class II presentations of that epitope should be able to antagonize any T-cell clone specific for that epitope. In other words, if an APC with typically ~100,000 MHC molecules, took up, processed, and presented 100 molecules from a PM library, it should present at least ten antagonistic peptides for any antigen-specific T-cell clone it encountered! If T-cell antagonism is a trans-dominant effect in which a relatively small number of antagonistic MHC/peptide complexes on an APC can prevent T-cell activation even in the presence of larger amounts of antigenic peptide, then, given the capacity of professional APCs to take up, process, and present large amounts of protein, it seems reasonable to expect that a moderate dose of a conservative 1-hit-per-10-mer PM library would equip most APCs to neutralize a substantial fraction of the inflammatory T-cell repertoire, leading to significant amelioration of symptoms, and possibly a substantial reduction of the repertoire by anergy or apoptosis.

In the examples it will be described how to construct and test a tolerogenic PM library of a test antigen. However, the antigen used here is only an example and the same methods may also be used to construct a tolerogenic PM library of any inflammatory self antigen. Other relevant autoantigens for which PM libraries can be generated are acetylcholine receptor,

collagen type II and insulin. PM libraries of these autoantigens will result in pharmaceutical compositions for the treatment of myestenia gravis, rheumatoid arthritis and insulin dependent diabetes mellitus, respectively.

The test antigen is myelin basic protein (MBP) and the library, with a modal frequency of one conservative mutation per ten amino acids, will be expressed in E. coli and the protein will be purified. APCs charged in vitro with the mutant MBP library will be compared with wildtype (wt) MBP-charged APCs for their ability to activate monoclonal T-cells specific for one or more immunodominant epitopes of MBP. The library will then be titrated against wt MBP to test for its ability to antagonize T-cell activation. Finally, the library will be tested for its ability to antagonize disease induction by wildtype MBP in the Lewis rat model of EAE.

EXAMPLES

EXAMPLE 1

Cloning of recombinant rat MBP

Poly A-containing RNA from the brains of male Sprague-Dawley rats was obtained commercially, and was reverse-transcribed into first-strand cDNA with oligo-dT primers. The full-length coding sequence of the 129-amino acid Myelin Basic Protein (MBP) was recovered from the cDNA by PCR using primers shown in Figure 1, designed from the Genbank cDNA sequence for rat MBP (M25889, Schaich et al., 1986, Biol. Chem. Hoppe-Seyler 367, 825-834). The 411 bp product was digested with NdeI and XhoI, gel purified, and ligated into the T7 expression vector pET23a (Novagen, Madison, WI, USA), similarly digested. This vector is an ampicillin-resistant phagemid with a pBR322 replicon, and expresses an open reading frame from the NdeI site. Transcripition is driven by the powerful phage T7 promoter and the T7 RNA polymerase, which must be supplied by the host. Six histidines are encoded immediately downstream from the XhoI site to provide a C-

terminal His tag for convenient one-step purification by Immobilized Metal-ion Affinity Chromatography (IMAC). The ligation product was introduced into competent cells of the E. coli strain XL-1 Blue by Ca^{++}-mediated transformation, and transformants were selected for ampicillin resistance on solid medium. Plasmid DNA was prepared from several clones by the alkaline lysis method and sequenced from the T7 promoter to the T7 terminator to confirm the integrity of the expression cassette and the MBP coding sequence.

EXAMPLE 2

Introduction of encephalitogenic T77S mutation into rat MBP

The principal epitope involved in EAE (Experimental Autoimmune Encephalomyelitis) in the Lewis rat system is between S69 and F86 (Offner et al., 1989). This fragment from guinea pig MBP is encephalitogenic when injected into Lewis rats, and its only difference from the rat MBP fragment is Ser instead of Thr at position 77 of rat MBP. So, to create a recombinant encephalitogenic MBP we introduced this mutation into the wildtype rat MBP. This was accomplished by amplifying the entire wt MBP expression construct by inverse PCR using a mutagenic primer and a head-to-head companion. The wildtype ACC codon for Thr77 was changed to TCC for Ser. A unique restriction site was also needed at the ends of the primers for closure. We used a computer programm known as 'GMAP program' to locate sites near the mutation which could be changed to encode a convenient restriction site without changing the encoded amino acid sequence. A unique SalI site was created at Val84-His85. This allowed to encode the Thr77-to-Ser mutation (also indicated as T77S mutation) in the antisense primer as shown in Figure 2. Inverse PCR was carried out with these primers using the rat MBP construct as template and the long range kit from Boehringer Mannheim. After digestion with SalI and ligation, XL-1 Blue cells were transformed

as above, and plated on ampicillin. Plamid miniprep DNA from several clones was sequenced as above to confirm the intended mutations and none other.

EXAMPLE 3

Construction of PM library of one- and two-hit mutants of the encephalitogenic epitope of MBP

The encephalitogenic CD4+ T-cell clone MBP1 is specific for L72-V85 in the guinea pig MBP, which is equivalent to L70-V83 (Chou etl, 1989 and Offner et al, 1989) in the rat MBP with S77 instead of T. Thus, rat L70-V83 was mutagenized. We wanted to maximize the frequency of clones with one or two hits per nine sites as this is the typical length of TcR-contacting peptide.

Using PM-CAD, it was computed that a codon doping rate of 1.43 substitutions per nine residues gives 63% of one-mutation and two-mutation and only 21% wild-type. This means that the nucleotide substitution frequency must be set to give an amino acid substitution frequency of 16% at each position. Next, using PM-CAD doping codons were selected for each position in the rat L70-V83 epitope on the basis of a user-specified set of alternative amino acid. The program extracts all doping codons which encode the minimum set from a database and computes the frequency in the specified library of each encoded alternate amino acid at the position in question based on the specified modal mutation frequency, in this case one per nine amino acid. Since we wanted our substitutions to be as conservative as possible, doping codons were selected which encode only the two or three most conservative analogs for each amino acid. Once doping codons were selected for each parental amino acid, the PM-CAD program computed the nucleotide mixtures for oligonucleotide synthesis.

The primer extends 20 bases beyond L70 to provide a good stretch of perfect match for priming.

The mutagenic strategy is shown in Table 2 with the parental amino acid sequence (pAA) and associated

codons, the doping codons (Dope), and the set of substitute amino acids for each position (Subs). Also shown are the frequencies (F) of each parental and substitute amino acid at each position.

Table 2

		F		F		F		F		F		F		F
pAA=>	L70	0.841111	P71	0.841111	Q72	0.841111	K73	0.841111	S74	0.841111	Q75	0.841111	R76	0.841111
Code=>	CTT		CCT		CAG		AAG		TCT		CAG		AGG	
Dope=>	NNT		NNT		NNK		NNK		NNT		NNK		NNG	
Subs=>	F	0.025337	S	0.025337	H	0.049939	N	0.049939	F	0.025337	H	0.049939	W	0.025337
	P	0.025337	L	0.025337	X	0.016646	Q	0.016646	Y	0.025337	X	0.016646	R	0.025337
	H	0.025337	H	0.025337	L	0.016646	X	0.016646	C	0.025337	L	0.016646	M	0.025337
	R	0.025337	R	0.025337	P	0.016646	M	0.016646	P	0.025337	P	0.016646	T	0.025337
	I	0.025337	T	0.025337	R	0.016646	T	0.016646	T	0.025337	R	0.016646	K	0.025337
	V	0.025337	A	0.025337	K	0.016646	R	0.016646	A	0.025337	K	0.016646	G	0.025337
					E	0.016646	E	0.016646			E	0.016646		
pAA=>	T77	0.841111	Q78	0.841111	D79	0.841111	E80	0.841111	N81	0.841111	P82	0.841111	V83	0.841111
Code=>	ACG		CAG		GAT		GAG		AAT		CCT		GTT	
Dope=>	NNG		NNK		NNK		NNK		NNK		NNT		NNT	
Subs=>	S	0.025337	H	0.049939	E	0.049939	D	0.049939	K	0.049939	S	0.025337	F	0.025337
	P	0.025337	X	0.016646	Y	0.016646	X	0.016646	Y	0.016646	L	0.025337	L	0.025337
	M	0.025337	L	0.016646	H	0.016646	Q	0.016646	H	0.016646	H	0.025337	I	0.025337
	K	0.025337	P	0.016646	N	0.016646	K	0.016646	I	0.016646	R	0.025337	A	0.025337
	R	0.025337	R	0.016646	V	0.016646	V	0.016646	T	0.016646	T	0.025337	D	0.025337
	A	0.025337	K	0.016646	A	0.016646	A	0.016646	S	0.016646	A	0.025337	G	0.025337
			E	0.016646	G	0.016646	G	0.016646	D	0.016646				

TABLE 2

The mutagenic primer and the sense primer from example 2 was used to amplify the entire expression construct by inverse PCR as was done for the T77S mutagenesis. The product was cut with Sal I and ligated as above. The ligation product was cleaned up and introduced into XL-1 Blue cells by high-voltage electroporation. 10^5 transformants were obtained from 18 ng of ligation DNA using cells that gave 108 transformants/ug using pure plasmid. Thus, ~5% of the library DNA was ligated and transformation competant. Cells were then transformed with ~1 ug of ligation product, yielding ~10^6 independent transformants. After recovery, the cells were diluted and grown to OD_{600} ~0.7 and treated with chloramphenicol (34 ug/ml) overnight. Plasmid DNA was purified representing the 10^6 independent clones. The MBP coding sequence was recovered from 20 independent clones by colony PCR and cycle sequenced to confirm the distribution of mutations.

EXAMPLE 4

Expression and purification of recombinant wild-type rat MBP, T77S mutant rat MBP and rat MBP PM library

The expression constructs, prMBP and prMBPT77S, and the prMBP PM library were introduced as pure plasmid DNA into E.coli strain BL21(DE3)/pLysS by high-voltage electroporation for high-level expression. Individual clones were examined for expression by running extracts on SDS-PAGE and silver staining. Expression of MBP appeared to be quite toxic to E. coli, but was completely suppressed in this host in the absence of induction. After 3 hrs of induction with 1mM IPTG, all clones accumulated an abundant protein of the expected size (~15 kDa) in amounts equal to at least 10% of total cell protein. This is equivalent to approximately 5 mg/liter at an OD_{600} of 1, more than enough for the proposed studies.

MBP protein was then purified from cultures of each of the three constructs by metal ion affinity

chromatography. The cells were harvested and lysed in buffered 6M guanidine HCl. The supernatant was then equilibrated with a 50% slurry of Ni-NTA resin (Qiagen, Chatsworth, USA) and poured into a column. The column was then washed with buffered 8 M urea until the A_{280} of the flow-through was <0.01. The protein was then eluted with mild acid (pH 5.9, then pH 4.5), and fractions were monitored by SDS-PAGE. The appropriate fractions were pooled and dialyzed against PBS containing 10% glycerol. The final yield was determined by the Pierce BCA assay (Pierce, Rockford, USA) and purity was affirmed by SDS-PAGE and silver staining.

EXAMPLE 5

Effects of the rat MBP PM library on monoclonal and polyclonal T cells in vitro

The CD4+ T-cell clone MBP1, specific for the positions 72-85 of GP-MBP was isolated from the draining popliteal lymphnodes of a Lewis rat 9 days after immunization into each hind footpad with 50µl guinea pig MBP (10µg) in Complete Freund's Adjuvant (CFA) (4mg/ml Mt). To maintain the T-cell clone, cells were cyclically restimulated in Iscove's modified Dulbecco's medium (Gibco) supplemented with 2% normal Lewis rat serum, 2mM L-glutamine, 2-ME and antibiotics, for 3-4 days, with irradiated (3000 rads) thymocytes as Antigen Presenting Cells (APCs) and 10 µg/ml MBP. After restimulation T-cells were propagated for 6 or 7 days in Iscove's Modified Dulbecco's Medium (Gibco) supplemented with 10% Fetal Calf Serum (FCS), 10% EL-4 supernatant (IL-2 source), 2 mM L- glutamine, 2-mercaptoethanol (2-ME), antibiotics and 1% non-essential amino acids.

The ability of the purified wild-type rat MBP, the T77S mutant rat MBP and the rat MBP PM library to stimulate the T-cell clone MBP1 was tested in a proliferation assay. Proliferative responses are measured in flat-bottom microtiter plates in triplicate cultures. Each well contains $2x10^4$ T-cells suspended in

0.2 ml Iscove's Modified Dulbecco's Medium supplemented with 2% Lewis rat serum, 2 mM L-glutamine, 2-ME and antibiotics in the presence of various amounts of antigens and irradiated (3000 rads) syngeneic thymocytes ($1x10^6$/well) as APCs. T-cells are cultured for 3 days and pulsed for 18 hours with [^{3}H]-thymidine. Cells are harvested on fiberglass filters and [^{3}H]-thymidine incorporation is measured.

The ability of various concentrations of the rat MBP PM library to induce proliferation of clone MBP1 is compared with the ability of the purified wild-type rat MBP and the T77S mutant rat MBP.

Also the capacity of the rat MBP PM library to induce IL-2 and IFNγ production by clone MBP1 is measured and compared to purified wild-type rat MBP and the T77S mutant rat MBP. MBP1 normally produces IL2 (measured by CTLL16 bioassay) and IFNγ (measured by rat-specific IFNγ ELISA) upon MBP stimulation. By using RT-PCR the induction of MRNA for IL4, IL10, IL2, IFNγ and TGF-ß is checked. Rat specific cytokine PCR primers based on published rat cytokine genes are available.

Furthermore, whether the rat MBP PM library induces proliferation of MBP1 or not, in both cases, the effect of a subsequent stimulation with the T77S mutant rat MBP is evaluated in terms of nonresponsiveness (decreased proliferation upon restimulation with the T77S mutant rat MBP, and changed cell surface expression of TCR/CD3, IL2R, and CD4 with flow cytometry analysis), apoptosis (quantitation of DNA strand breaks by using terminal transferase (TdT) to label free 3'OH ends in genomic DNA with fluorescein-dUTP, analysis by flow cytometry), and altered cytokine production. If the rat MBP PM library is not able to induce proliferation of clone MBP1, it is also tested whether the library has MHC-blocking effects.

To assess the capacity of the rat MBP PM library to inhibit antigen-induced proliferation of clone MBP1, varying concentrations of the library are added to

the culture containing T-cells and irradiated thymocytes 2 h before the addition of a suboptimal dose of T77S mutant rat MBP. Alternatively, a fixed concentration of rat MBP PM library and a concentration range of T77S mutant rat MBP are tested as well. To distinguish between MHC-blockade and antigen specific effects of the rat MBP PM library, inhibition of antigen-induced proliferation of T-cell clones specific for e.g. ovalbumin or mycobacterial 65 kD heatshock protein, which recognize their specific epitopes in the context of the same MHC restriction element (RT1.B1) as clone MBP1, is tested as well. The T lymphocyte proliferation assay is performed as mentioned above. In case where the antigen-induced proliferation of MBP1 is inhibited more efficiently than the proliferation of the unrelated T-cell clones, the antigen-specific modulatory effect of the mutant MBP library is further analyzed.

Antagonistic effects of the rat MBP PM library on T-cell clone MBP1 are evaluated as described by DeMagistris et al. (1992). In short, APCs is pulsed with suboptimal doses of stimulatory peptide MBP72-85 or MBP for 2-4 hrs., unbound antigen is removed by washing, rat MBP PM library is added together with the MBP1 T cells and after 3 days T-cell proliferation is determined by ^{3}H-thymidine incorporation. Production of IL-4, IL-2, IFNγ, IL-10, TGF-ß, nonresponsiveness, and apoptosis is also evaluated. Since it has been reported that antagonistic epitopes present on different targets from the wt-epitope could inhibit lysis of the wt-epitope presenting cells (Klenerman et. al., 1994), the ability of rat MBP PM library-pulsed APC to inhibit the induction of proliferation by T77S mutant rat MBP-pulsed APC is also tested. Besides all experiments performed with clone MBP1, lymph node cells of rats immunized with MBP/CFA are isolated and subjected to the same experiments.

EXAMPLE 6

The ability of the rat MBP PM library to activate the encephalitogenic capacity of MBP specific T-cells in vitro

Lymph node cells of rats immunized with MBP/CFA are isolated and in vitro restimulated with WT-MBP or rat MBP PM library for 2 cycles. The capacity to passively transfer EAE to naive lewis rats is tested. Clinical signs of EAE are monitored daily on a scale of 0 to 4; 0, no signs; 0.5, weight loss; 1, limp tail; 2, hind leg weakness; 3, paraplegia; 4, paraplegia with forelimb weakness, moribund condition.

EXAMPLE 7

Test of the ability of the rat MBP PM library to antagonize MBP induction of experimental autoimmune encephalomyelitis (EAE) in a rodent model

Active EAE is induced by injecting 50µl of a 1:1 emulsion of GP-MBP or the encephalitogenic peptide GP-MBP72-85 in PBS (1mg/ml) and CFA (4 mg/ml Mt) in each hind footpad. Disease severity is scored as described above. First, the disease-inducing capacity of the rat MBP PM library is tested in a concentration dependent way. In cases where the library is not encephalitogenic, it is tested in vivo by co-immunization during induction of EAE with wtMBP. The rat MBP PM library is added in different concentrations to the MBP/CFA or MBP72-85/CFA disease-inducing emulsions, and the effects on clinical signs is evaluated. In cases where the rat MBP PM library is protective, also a fixed concentration of it and a concentration range of WT-MBP is tested to determine the strength of the inhibitory effect.

EXAMPLE 8

Generation and selection of a pharmaceutical composition for the treatment of multiple sclerosis from a Parsimonious Mutagenesis library of human myelin basic protein with modal frequency of ~1 mutation per 10 amino acid.

Myelin basic protein is generally accepted as a candidate autoantigen in multiple sclerosis. It has been shown that the region of amino acid position 84 to 106 represents the immunodominant region in the context of the multiple sclerosis associated HLA-DR alleles (Ota et al, 1990). However, it can not be excluded that other regions of the 21.5 kDa myelin basic protein contain epitopes that are recognized by disease promoting T cells in multiple sclerosis patients as well. Therefore, the whole human myelin basic protein sequence is used as the parental sequence for the generation of a PM libraries to maximize the number of possible disease-inducing epitopes covered. Peptides of the immunodominant region of human myelin basic protein described above bind with high affinity to multiple sclerosis associated HLA-DR alleles, in particular to both polymorphic alleles co-expressed in the DR15 Dw2 haplotype (HLA DRα paired with the product of HLA-DRB1*1501 = DR2b and HLA DRα paired with the product of HLA-DRB5*0101 = DR2a). These MHC class II molecules have the strongest association with multiple sclerosis and are therefore being targeted as first.

However, the same procedure can be used to generate and select for pharmaceutical compositions to treat multiple sclerosis patients with other HLA-DR phenotypes. The goal of the generation of human myelin basic protein PM libraries is to identify and select a population of human myelin basic proteins that can influence the activation of T cells. This influence can lead to complete abolishment of T-cell activation, to self inactivation of the T-cells or to the induction of suppression factors, like particular cytokines. The induction of suppression factors will ultimately lead to

downregulation of the immune response in total. Thus, the influence may also comprise a positive, upregulating or stimulating effect on suppressor T-cells.

It is expected that the most active population of human myelin basic proteins obtained from the PM library, fractions thereof, a set of species thereof, an individual species thereof or a part of such species is able to influence the activation of T cells in multiple sclerosis patients with the results described above, leading to a lasting amelioration of clinical symptoms and possible cure of the disease.

Below it is described how such populations of human myelin basic protein are generated in a PM library and what procedures and methods are used in the selection of the most active fraction, the most active set of species thereof, the most active individual species thereof or the most active part of such species, of the population of human myelin basic proteins obtained from the PM library.

The first step in PM library design is to decide on a scanning rate, or amino acid substitution frequency, which would allow a thorough search of the protein sequence space without sacrificing desirable properties. In this case, we wish to alter but not obliterate specific T-cell recognition of the human myelin basic protein, with minimal disturbance of MHC binding. Since MHC class II molecules typically present peptides of 8-12 amino acid, and most of the most active known antigen analog inhibitors of T-cell activation differ conservatively at only one or two positions from the parent antigen, it is decided that a modal substitution frequency of one per ten amino acids ensures the highest frequency of antagonists in the library. Human myelin basic protein contains 172 amino acids, encoded by 516 nucleotides. The entire human myelin basic protein sequence is mutagenized in ten PM libraries. In each successive library, 26 contiguous codons are mutagenized at the same rate of substitutions. Using PM-

CAD, a maximum possible frequency of one-mutation and two-mutation variants at 63% of each library is computed. This means that the nucleotide substitution frequency must be set to give an amino acid substitution frequency of 16% at each position. Each of the ten libraries overlaps with its neighbors by ten codons. This ensures that every possible nine-residu T-cell epitope will be contained in its entirety within at least one library.

Next, doping codons are selected for each position in the human myelin basic protein on the basis of a user-specified set of alternative amino acids. The PM-CAD program extracts all doping codons which encode the minimum set from a database and computes the frequency in the specified library of each encoded alternate amino acid at the position in question based on the specified modal mutation frequency, in this case one per ten amino acid. Since we want our substitutions to be as conservative as possible, doping codons are selected which encode only the two or three most conservative analogs for each amino acid. Once doping codons are selected for each parental amino acid, the PM-CAD program computes the nucleotide mixtures for oligonucleotide synthesis for each of the ten human myelin basic protein PM libraries.

Each of the ten mutagenic coding sequences of human myelin basic protein is reassembled from pairs of overlapping oligonucleotides. The members of each pair encode opposite strands and overlap at their 3' ends, so that each pair is converted to a continuos duplex by overlap extension PCR. Each of these duplexes overlaps its neighbors, so that equimolar mixtures are used to propagate the assembly. The oligos are assembled in a single pot reaction with an equimolar mixture of all oligos. The terminal oligos have convenient restriction sites for insertion of the product into the expression vector. Randomly annealing oligos will have on average one mismatch per 30 bp, which should not interfere with PCR except when they are near 3' ends. Thus, there is a

bias against mutations near the 3' ends of the oligos and this is minimized by using low annealing temperatures. The human myelin basic protein PM libraries are expressed and purified as described above in Example 4.

Each of the human myelin basic protein PM libraries is tested with T-cells or T-cell clones from multiple sclerosis patients. Human myelin basic protein-specific T-cell clones are generated by the split well technique (Martin et al, 1991) starting with peripheral blood mononuclear cells (PBMC) from multiple sclerosis patients. The T-cell clones are tested for induction of proliferation by culture in U-bottom 96-well plates (10,000/well) using irradiated (3000 rad) PBMC (50,000/well) as APC and various concentrations of purified protein from the human myelin basic protein PM libraries. Cells are cultured for 3-5 days at 37 °C and during the last 6 hours of the culture, [^{3}H]thymidine is added to each well.

The cells are harvested and incorporated radioactivity was measured by scintilation counting. The capacity of the human myelin basic protein PM libraries to induce T-cell anergy is performed as described (Sloan-Lancaster et al, 1993). Briefly, T-cell clones (2 x 10^5) and autologous irradiated PBMC (1 x 10^6) as antigen presenting cells are incubated at 37 °C alone or with 100 µg/ml of protein from the human myelin basic protein PM libraries for 48 h in a 24-well tissue culture plate. T cells are separated from the antigen presenting cells by sodium metrizoate gradient centrifugation, washed twice, rested for 2 days and than challenged in a 72-h proliferation assay using 1 x 10^4 T cells/well together with 5 x 10^4 irradiated PBMC and different concentrations of wild-type human myelin basic protein.

In some experiments, exogenous IL-2 (20 U/ml) is added during the challenge period. TCR antagonism is evaluated in a prepulse-wash assay as described (De Magistris et al, 1992). Autologous irradiated (3000 rad) peripheral blood or EBV-transformed ^{51}Cr-labeled B cells

are incubated with suboptimal concentrations wild-type human myelin basic protein in complete medium at 1 x 10^6 - 2 x 10^6 cells/ml for 3-4 h at 37 °C. PBMC and B cells are then washed and adjusted to 1 x 10^6 and 1 x 10^5 cells/ml, respectively. Of these suspensions, 50 µl is added to 96-well U-bottom plates containing 1 x 10^4 T cells for proliferative analysis. Different concentrations of the human myelin basic protein PM libraries are added in 50 µl medium and assays then carried out as described (De Magistris et al, 1992).

For the generation of supernatants, 2 x 10^5 T cells are seeded in 24-well plates together with 1 x 10^6 irradiated autologous PBMC in 1 ml complete medium in the presence or absence of 10 µg/ml of wild-type human myelin basic protein or protein from the human myelin basic protein PM libraries. Supernatants are collected after 24 or 72 h and stored at -70°C until the assays are performed. For the TGF-ß experiments, antigen presenting cells (autologous PBMC or autologous EBV-transformed B cells) are pre-incubated for 2 h with 200 µg/ml mytomicin C (Sigma, St. Louis, MO), washed three times and seeded together with T cells. IFNγ, IL-4 and TGF-ß are measured using commercial ELISA (Biosource, Camarillo, CA for IFNγ and IL4; R&D Systems, Minneapolis, MN for TGF-ß, respectively).

For each T-cell clone, one or two libraries are identified that can influence its activation. The desired influence on the activation of the T-cell clones by the PM libraries is defined as having the capacity to induce T-cell anergy tested as described above, having TCR antagonism activity tested as described above or a shift in the cytokine profile tested as described above. One library that can influence the activity of one particular T-cell clone is identified when at least some necessary T-cell epitope residues fall within the six-residue non-overlap region, defining the epitope to within 22 residues, i.e., the six non-overlapping residues plus up to eight on either side. Two libraries that can influence

the activity of one particular T-cell clone are identified when the T-cell epitope falls within an overlap region of the two libraries. In this case the epitope is defined within ten amino acid residues and does not need to be further defined (average length of T-cell epitope presented in the context of MHC class II molecules is nine amino acids). The 22-residue epitopes are further deconvoluted with two overlapping 14-residue libraries and a 16 residue library spanning the middle. These allow the epitope to be defined to within 11 amino acid residues.

Once the epitope for each myelin basic protein-specific T-cell clone is defined, the library for each defined epitope is deconvoluted to identify amino acid substitutions that result in epitopes that can influence the activity of the T-cell clone. This is accomplished by making pools of all 45 possible 2-mutation permutations of each 10-residue epitope. These libraries include all relevant mutations because in addition to all two-side mutants, all one-side mutants are also present in each library, and many active variants with more than two mutations are not expected. Each selected pair of sites is then deconvoluted to test each site seperately and again each substitution is tested at each site either alone or in the pair. This results in a rank order of the most potent one- and two-mutation analogs for each T-cell epitope.

If using the panel of T-cell clones epitopes are mapped in different libraries, the most potent one- and two-mutation analogs for each T-cell epitope can be combined in one ore more protein species for therapeutic use in multiple sclerosis patients.

REFERENCES

1. Allen PM. 1994. Cell 76, 593-596.
2. Balint RF, and Larrick JW. 1993. Gene 137, 109-118.
3. Bertoltti A, et al. 1994. Nature 369, 407-410.
4. Brocke S, et al. 1996. Nature 379, 343-346.
5. Chien Y-h, and Davis MM. 1995. Immunology Today 14, 597-602.
6. Chou et al. J. Neurosci. Res. 22, 181-187.
7. De Magistris MT, et al. 1992. Cell 68, 625-634.
8. Elias D, et al. 1995. Eur. J. Immunol. 25, 2851-2857.
9. Evavold BD, et al. 1993. Immunology Today 14, 602-609.
10. Evavold BD and Allen PM. 1991 Science 252, 1308-1310.
11. Fairchild PJ, and Wraith DC. 1996. Immunology Today 17, 80-85.
12. Franco A, et al. 1994. Eur. J. Immunol. 24, 940-946.
13. Jameson SC, et al. 1993. J. Exp. Med. 177, 1541-1550.
14. Janeway CA Jr. 1995. Immunology Today 16, 223-225.
15. Klenerman P, et. al. 1994. Nature 369:403-407.
16. Kuchroo V, et al. 1995. Cell 80, 707-718.
17. Moffat BA, and Studier FW. 1986. J. Mol. Biol. 189, 113-130.
18. Munn DH, et al. J. Immunol. 156, 523-532.
19. Nagata S, and Golstein P. 1995. Science 267, 1449-1456.
20. Offner et al., J. Exp. Med. 170, 355-362.
21. Ostrov D, et al. 1993. J. Immunol. 150, 4277-4283.
22. Paul WE, and Seder RA. 1994. Cell, 76, 241-251.
23. Ridgway WM, et al. 1994. Curr. Op. Immunol. 6, 946-955.
24. Ruppert J, et al. 1993. Proc. Natl. Acad. Sci. USA, 90, 2671-2675.
25. Sakihama T, et al. 1995. Immunology Today 16, 581-587.
26. Schaich et al., 1986, Biol. Chem. Hoppe-Seyler 367, 825-834

27. Sette A, et al. 1994. Annu. Rev. Immunol. 12, 413-431.
28. Sloane-Lancaster J, et al. 1993. Nature 363, 156-159.
29. Valitutti S, et al. 1995. Nature 375, 148-151.
30. Yoon ST, et al. 1994. Immunity 1, 563-569.
31. Martin R, et al. 1991. J. Exp. Med. 173, 19-27.
32. Ota K. et al. 1990. Nature 346, 183-185.

CLAIMS

1. Population of protein molecules having a distribution of specific mutations in the amino acid sequence as compared to a parent protein.

2. Population of protein molecules as claimed in claim 1, obtainable by establishing the parental amino acid sequence of the protein to be mutated and deriving therefrom a parental nucleotide sequence encoding the amino acid sequence, optionally selecting sites within this parental amino acid sequence, mutation of which is more desirable or less desirable, designing overlapping oligonucleotides encoding parts of the protein and harbouring one or more codon changes as compared to the corresponding parental nucleotide sequence, composing of the overlapping oligonucleotides a population of mutated nucleotide sequences encoding mutated versions of the parent protein thus establishing a library, and expressing the mutated versions of the mutated nucleotide sequences to obtain a population of mutated proteins.

3. Population of protein molecules as claimed in claims 1 and 2 for use in the induction of immunological tolerance of the parent protein.

4. Population of protein molecules as claimed in claim 3, wherein the induction of immunological tolerance is used for treatment and/or prevention of immune-related disorders, such as autoimmune diseases or allergic diseases.

5. Method for producing a population of protein molecules as claimed in claims 1-4, comprising establishing the parental amino acid sequence of the protein to be mutated and deriving therefrom a parental nucleotide sequence encoding the amino acid sequence, optionally selecting sites within this parental amino acid sequence, mutation of which is more desirable or less desirable, designing overlapping oligonucleotides encoding parts of the protein and harbouring one or more codon changes as compared to the corresponding parental

nucleotide sequence, composing of the overlapping oligonucleotides a population of mutated nucleotide sequences encoding mutated versions of the parent protein thus establishing a library, and expressing the mutated versions of the mutated nucleotide sequences to obtain a population of mutated proteins.

6. Method as claimed in claim 5, further comprising selecting a fraction, set of species, individual species or part of a species having T-cell activation antagonising activity from the population.

7. Method as claimed in claim 7, wherein the selection comprises studying the ability of the whole population, fractions, sets of species, individual species or parts of species to antagonise the activation of T-cells, either in vitro or in vivo and is performed by contacting monoclonal or polyclonal T-cells specific for the parent protein from which the population of mutated proteins is derived with the population, fraction, set of species, individual species and/or part of a species.

8. Fraction of a population as claimed in claims 1-4, obtainable by separating the population on the basis of a criterion.

9. Fraction as claimed in claim 9, which fraction is obtainable by separating the population of claims 1-4 based on having agonizing or antagonizing activity on T-cell activation.

10. Set of species of a population as claimed in claims 1-4 or a fraction as claimed in claims 8 and 9, obtainable by separating the population or the fraction on the basis of a criterion.

11. Set of species as claimed in claim 10, which fraction is obtainable by separating the population of claims 1-4 or the fraction as claimed in claims 8 or 9 based on having agonizing or antagonizing activity on T-cell activation.

12. Individual species of a population as claimed in claims 1-4 or a fraction as claimed in claims 8 or 9

or a set of species as claimed in claims 10 or 11, obtainable by separating the population or the fraction or the set of species on the basis of a criterion.

13. Individual species as claimed in claim 12, which set is obtainable by separating the population of claims 1-4 or the fraction of claims 8 or 9 or the set of claims 10 or 11 based on having agonizing or antagonizing activity on T-cell activation.

14. Part(s) of one or more individual species as claimed in claims 12 and 13, obtainable by separating an individual species into one or more parts on the basis of a criterion.

15. Part(s) as claimed in claim 14, which part(s) is(are) obtainable by separating an individual species of claims 12 or 13 based on having agonizing or antagonizing activity on T-cell activation.

16. Use of one or more populations as claimed in claims 1-4 and/or one or more fractions as claimed in claims 8 or 9 and/or one or more sets of species as claimed in claims 10 or 11 and/or one or more individual species as claimed in claims 12 or 13 and/or one or more parts of one or more species as claimed in claims 14 or 15 for the preparation of a medicament having T-cell activation antagonising activity.

17. Use as claimed in claim 16, wherein the medicament is intended for the treatment and/or prophylaxis of autoimmune disorders, like rheumatoid arthritis, multiple sclerosis, type I diabetes, lupus, thyroiditis and myasthenia gravis.

18. Population of mutated human myelin basic protein, having one or more mutations in one or more epitopes.

19. Fraction of a population as claimed in claim 18.

20. Set of species selected from a population of claim 18 and/or a fraction of claim 19.

21. Individual species selected from a population of claim 18 and/or a fraction of claim 19 and/or a set of species of claim 20.

22. Fraction of an individual species of claim 21.

23. Population of claim 18 and/or fraction of claim 19 and/or set of claim 20 and/or individual species of claim 21 and/or fraction of a species of claim 22 for use in the treatment of multiple sclerosis.

24. Use of a population of claim 18 and/or fraction of claim 19 and/or set of claim 20 and/or individual species of claim 21 and/or fraction of a species of claim 22 for the preparation of a medicament for the treatment of multiple sclerosis.

```
                                  M  A  S  Q  K  R  P  S
Sense:     5'- GGC CTG GAT CAT ATG GCA TCA CAG AAG AGA CCC TCA C -3'
                       NdeI

                           E  L  R  R  A  M  P  S  G  S
Antisense:5'- GGG AGG CTC GAG GCG TCT TGC CAT GGG AGA TCC AGA G -3'
                       XhoI
```

FIGURE 1

```
              D79 E  N  P  V  V  H  F  F  K  N
Sense:     5'- GAT GAA AAC CCA GTA GTC GAC TTC TTC AAG AAC -3'
                                   SalI

              K88 F  F  H  V  V  P  N  E  D  Q  S77 R  Q  S
Antisense: 5'- CTT GAA GAA GTC GAC TAC TGG GTT TTC ATC TTG GGA CCT CTG CGA C -3'
                       SalI
```

FIGURE 2

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 97/00777

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/01 C07K14/47 A61K38/17

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	GENE, vol. 137, no. 1, 27 December 1993, AMSTERDAM NL, pages 109-118, XP002031537 ROBERT F. BALINT ET AL.: "Antibody engineering by parsimonious mutagenesis" cited in the application see page 111, right-hand column, paragraph 3 - page 117, left-hand column, paragraph 2 ---	1,2,5
X	WO 95 22625 A (AFFYMAX TECHNOLOGIES) 24 August 1995 see page 5, line 36 - page 10, line 1 --- -/--	1,2,8, 10,12,14

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 August 1997	18.08.97

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Montero Lopez, B

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Interr ial Application No
PCT/EP 97/00777

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MOL. GENET. DEV. NEUROBIOL., TANIGUCHI SYMP. BRAIN SCI., 9TH (1986) , 1986, pages 111-123, XP000672601 ROACH, ARTHUR ET AL.: "Normal and mutant genes for the mouse myelin basic proteins" see page 112, paragraph 2 - page 122, paragraph 1 ---	18-24
A	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 87, no. 4, February 1990, WASHINGTON US, pages 1337-1341, XP000103572 VIPIN KUMAR ET AL.: "Amino acid variations at a single residue in an autoimmune peptide profoundly affect its properties: T-cell activation, major histocompatibility complex binding, and an ability to block experimental allergic encephalomyelitis " see abstract see page 1337, left-hand column, paragraph 2 - right-hand column, paragraph 1 see page 1338, left-hand column, paragraph 4 - page 1341, left-hand column, paragraph 2 ---	18-24
A	EUR. J. IMMUNOL., vol. 24, no. 5, 1994, pages 1053-1060, XP000672512 WAUBEN, MARCA H. M. ET AL.: "Inhibition of entire myelin basic protein-induced experimental autoimmune encephalomyelitis in Lewis rats by major histocompatibility complex class II-binding competitor peptides " see abstract see page 1053, right-hand column, paragraph 3 - page 1054, left-hand column, paragraph 2 see page 1055, left-hand column, paragraph 3 - page 1057, right-hand column, paragraph 1 ---	18-24
P,X	GENE (1996), 169(2), 147-55 CODEN: GENED6;ISSN: 0378-1119, 1996, XP002031538 SCHIER, ROBERT ET AL: "Identification of functional and structural amino-acid residues by parsimonious mutagenesis" see abstract see page 149, right-hand column, paragraph 2 - page 153, right-hand column, paragraph 1 --- -/--	1,2,5

2

INTERNATIONAL SEARCH REPORT

Inter nal Application No
PCT/EP 97/00777

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	WO 96 16085 A (NEUROCRINE BIOSCIENCES) 30 May 1996 see page 2, line 35 - page 3, line 16	18-24

2

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 97/00777

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

See Form PCT/ISA/210 (continuation sheet)

1. ☒ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☒ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/EP 97/ 00777

FURTHER INFORMATION CONTINUED FROM PCT/ISA/210

1. Claims 1-17. Population of protein molecules having a distribution of specific mutations as compared to a parent protein, method for its production; fraction thereof, set of species and individual species thereof, part of the individual species, and use of any of the foregoing for the preparation of a medicament having T-cell activation antagonising activity.

2. Claims 18-24. Population of mutated human myelin basic protein having one or more mutations in one or more epitopes, fraction thereof, set of species and individual species thereof, fraction of the individual species; and use of any of the foregoing for treatment of multiple sclerosis.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr al Application No
PCT/EP 97/00777

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9522625 A	24-08-95	US 5605793 A	25-02-97
		AU 2971495 A	04-09-95
		CA 2182393 A	24-08-95
		CN 1145641 A	19-03-97
		EP 0752008 A	08-01-97
WO 9616085 A	30-05-96	AU 4405796 A	17-06-96